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Genotypic diversity and antifungal susceptibility of *Cryptococcus neoformans* isolates from pediatric patients in China

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Summary

Cryptococcosis is a life-threatening mycosis primarily occurring in adult patients particularly those with immunosuppression such as HIV infection/AIDS. The number of reported cases of pediatric cryptococcosis has increased in the last decade around the world, including China. However, current information on the characteristics of cryptococcosis in children, particularly the genotypic diversity and antifungal susceptibility of the isolates, is limited. In the present study, a total of 25 pediatric isolates of *Cryptococcus neoformans* were genotyped using the ISHAM-MLST scheme. *In vitro* susceptibility to antifungal agents of the 22 isolates was tested using the CLSI M27-A3 method. Our analyses revealed that the genotypic diversity of *C. neoformans* isolates from Chinese pediatric patients was low, with ST 5 (80%) and ST 31 (12%) being the two major sequence types. Reduced susceptibility to fluconazole (FLU), 5-flucytosine (5-FC), and itraconazole (ITR) was observed among *C. neoformans* isolates from Chinese pediatric patients, particularly among the ST5 isolates, which was similar to observations made on *C. neoformans* isolates from Chinese adult patients. In addition, the majority of isolates (3/4, 75%) obtained from deceased patients showed decreased antifungal susceptibility, which indicates that further monitoring of antifungal susceptibility of *Cryptococcus* isolates is warranted in management of pediatric cryptococcosis.

Introduction

Cryptococcus neoformans and *Cryptococcus gattii* are two major basidiomycetes yeasts which form a species complex and are associated with cryptococcosis, a life-threatening mycosis involving both immunosuppressed and immunocompetent hosts.[1,2] *Cryptococcus neoformans* has a worldwide distribution, occurring naturally in avian excreta and causing over 80% of cryptococcosis cases each year.[3] *Cryptococcus gattii* is mainly associated with trees and has emerged as a pathogen of immunocompetent humans in temperate regions of North America.[4]

The recent development of molecular techniques, such as multi-locus sequence typing (MLST), has improved our understanding of the genetic diversity and taxonomy of *C. neoformans* and *C. gattii* as both are recognized as species complexes.[5-7] *Cryptococcus neoformans* (serotype A) has been divided into three major molecular types, namely AFLP1/VNI, AFLP1A/VNII/VNB, and AFLP1B/VNII; molecular type of *Cryptococcus deneoformans* (serotype D) is AFLP2/VNIV; molecular type of the hybrid isolates (serotype AD) is AFLP3/VNIII.[7] In addition, *C. neoformans* and *C. gattii* have two opposite mating types, *MAT*α and *MAT***a**, with *MAT*α being the prevalent mating-type of clinical and environmental isolates.[8,9]

The rapid emergence of multidrug-resistant pathogenic fungi has become a major threat to human health.[10] Currently, azoles, particularly fluconazole (FLU), are generally recommended for the treatment of cryptococcosis during the consolidation and maintenance phases.[11] However, cryptococcosis caused by FLU-resistant *Cryptococcus* isolates has recently been reported.[12] Moreover, antifungal susceptibility of *Cryptococcus* isolates has been noted to vary among genotypes and geographic regions.[1,5]

Cryptococcosis is primarily observed in adult patients with HIV/AIDS, while cases involving children are rarely reported.[13-16] It has been documented that pediatric cryptococcosis accounted for 0.9% of all cryptococcosis cases in South Africa.[17] The number of reported cryptococcosis in children, however, has increased worldwide in the last decade, including China.[16,18] A previous study indicated that the majority (approximately 70%) of children (>2 years old) in the Bronx, New York, USA had serologic evidence for *C. neoformans* infection,[19] particularly those older than 5 years old.[20]

To date, data on the characteristics of cryptococcosis in children, particularly the genotypic diversity and antifungal susceptibility of the isolates, is limited. To the best of our knowledge, only one study from South Africa has investigated isolates from pediatric cryptococcosis cases, indicating a high genetic diversity among *C. neoformans* isolates from pediatric patients. Here we report the first genotyping and antifungal susceptibility profiles of pediatric cryptococcosis in China.

Materials and methods

Isolates

The pediatric cryptococcosis cases in the present study were all confirmed by positive culture. Patients whose age at the time of diagnosis was <16 years were included in this study. Twenty-two *C. neoformans* strains isolated from pediatric patients were included in our study. All these isolates were collected from six university hospitals in China namely: Changzheng Hospital (n=15), Xinhua Hospital (n=1), and Huashan Hospital (n=1) in Shanghai; West China Hospital (n=2) in Chengdu, Sichuan Province; the First Affiliated Hospital of Zhejiang University (n=2) in Hangzhou, Zhejiang Province; and Guangzhou General Military Hospital (n=1) in Guangzhou, Guangdong Province. In addition, we also included the MLST sequence

and clinical information of three pediatric isolates (PU 18, PU 31, and PU 158) which were reported previously from Beijing[21] for comparison analyses.

Mating type

To determine the mating type of the *C. neoformans* isolates, PCRs were performed as previously described.[22] *Cryptococcus neoformans* strains CBS 10512 (*MAT***a**; AFLP1/VNI), CBS10515 (*MAT*α; AFLP1/VNI), and *C. deneoformans* strains CBS 10511 (*MAT***a**; AFLP2/VNIV), CBS 10513 (*MAT*α; AFLP2/VNIV) were used as controls.

DNA extraction, PCR and sequencing

DNA extraction of *C. neoformans* isolates was performed following the instructions of the UltraClean® microbial DNA isolation kit (MoBIO Laboratories, Carlsbad, CA, USA). Each locus (*CAP59*, *GPD1*, IGS1, *LAC1*, *PLB1*, *SOD1*, and *URA5*) in *C. neoformans* was amplified in a 25 μL PCR volume according to the ISHAM-MLST scheme.[23] PCR products were purified with Sephadex G-50 fine (GE Healthcare Bio-Sciences, Uppsala, Sweden) and were sequenced from both directions. Sequence reads were manually edited using SeqMan v8.0.2 (DNASTAR, Madison, WI, USA) and each MLST sequence was aligned using MUSCLE version 3.8.31[24] and concatenated by the FASconCAT program,[25] and then queried against the online MLST database (http://mlst.mycologylab.org) to assign allele type (AT) for each of the seven loci. The final sequence type (ST) for each isolate was determined based on the combined allelic assignments at the seven sequenced loci.

Genotyping

Principal component analysis (PCA) of the sequence data of *C. neoformans* isolates was performed using the Adegenet 2.1.1 package[26] for software R (version 3.4.4). Moreover, nucleotide diversity (π), number of polymorphic sites (S), average number of nucleotide differences per sequence (k), and average number of nucleotide substitutions per site between populations (D_{xy}) were calculated using DnaSP version 6.10.03.[27] The recent study on pediatric *C. neoformans* isolates from South Africa did not follow the full ISHAM-MLST scheme to analyze genetic diversity. However, among the 11 loci analyzed by them, five loci (*GPD1*, *LAC1*, IGS1, *PLB1* and *SOD1*) overlapped with the seven loci used in our study. For comparative analysis of the pediatric *C. neoformans* isolates between China and South Africa, the results of our genetic diversity analysis were all based on the five-locus sequence data. Comparisons of ST distributions between *C. neoformans* isolates from Chinese pediatric cases and that from Chinese adult cases were conducted using Fisher's exact test corrected with the Benjamini-Hochberg method for multiple comparisons. The ST information of *C. neoformans* isolates from 162 Chinese adult cases was collected from two previous studies by Dou *et al.*[21] (n=77) and Chen *et al.*[28] (n=85).

Phylogenetic analysis

We selected four representative *C. neoformans* isolates from Chinese pediatric patients representing four different STs identified here, five *C. neoformans* isolates from Chinese adult patients with five different STs, and twenty-one isolates from pediatric patients in South Africa to show the relationships among the different STs in a phylogenetic tree. Our phylogenetic tree was based on the five-locus sequence data (*GPD1*, *LAC1*, IGS1, *PLB1* and *SOD1*). The sequence data of these five loci were aligned individually using MUSCLE version 3.8.31[24] and concatenated by FASconCAT program.[25] The concatenated alignment

was then uploaded into MEGA7.0.9 to construct a phylogenetic tree using maximum likelihood (ML) and maximum parsimony (MP) methods with K2+G model and 1,000-replicate of bootstrap test, respectively.[29]

Antifungal susceptibility testing

To assess the *in vitro* antifungal susceptibility of the isolates, antifungal drugs amphotericin B (AMB), 5-flucytosine (5-FC), fluconazole (FLU), itraconazole (ITR), voricoazole (VOR), posaconazole (POS) and isavuconazole (ISA) were applied to the *Cryptococcus* isolates using the CLST M27-A3 protocol.[30] The minimum inhibitory concentration (MIC) values were determined after 72 h of incubation at 35°C. *Candida krusei* strain ATCC 6258 and *C. parapsilosis* strain ATCC 22019 were used as quality control (QC) strains. Based on the recommendation of previous studies, the epidemiological cut-off values (ECVs) of *C. neoformans* for 5-FC and FLU used in the present study were 8 mg/L; 0.25 mg/L for ITR, VOR and POS; 1 mg/L for AMB; and 0.12 mg/L for ISA respectively.[31-33] In this study, isolates with MIC values above the ECVs were considered as non-wild-type (non-WT) isolates and isolates with MIC values equal to the ECVs were considered as having decreased susceptibility to antifungal drugs.

Results

Demographic data of the pediatric isolates

The isolates included in our study were collected from 25 pediatric patients living in 13 different provinces across China as shown in Table 1. The majority of the isolates were obtained from Sichuan (16%, 5/25), followed by Shanghai (12%, 3/25), Hubei (12%, 3/25), Beijing (8%, 2/25), Hebei (8%, 2/25), Henan (8%, 2/25), Shanxi (8%, 2/25), Zhejiang (8%, 2/25), Fujian (4%, 1/25), Anhui (4%, 1/25), Guangdong (4%, 1/25), Hunan (4%, 1/25), and

Jiangsu (4%, 1/25). The majority of these isolates were recovered from cerebrospinal fluid (CSF) (80%, 20/25), followed by blood (12%, 3/25), and skin (8%, 2/25). The mean age of these patients was 10.4 ± 3.5 years (range: 3-15 years), with the majority within the age range of 11-15 years (56%, 14/25). The male/female gender ratio was 1.5 (15:10). The majority of the pediatric patients (80%, 20/25) had no underlying diseases. Only three pediatric patients were HIV-infected, and two patients had systematic lupus erythematous (SLE). Patient characteristics are presented in Table 1 and Figure 1.

Genotypes and mating types

All of the pediatric isolates from China were identified as *C. neoformans* (AFLP1/VNI, *MAT*α). Among these, four STs were identified with ST5 (80%, 20/25) being predominant, followed by ST31 (12%, 3/25), ST53 (4%, 1/25), and ST93 (4%, 1/25). The details are shown in Table 1.

Phylogenetic analysis

The phylogenetic tree revealed that the predominant ST5 isolate (based on the ISHAM-MLST) in Chinese pediatric isolates (represented by strain SCZ 20172) clustered together with a ST9 isolate (based on 11-loci MLST) from the South Africa study (represented by strain RSA 1162). Moreover, the major ST8 isolate (based on 11 loci MLST) from South Africa (represented by strain RSA 1195) clustered together with ST 93 isolate (based on the ISHAM-MLST) from China (represented by strain SCZ 20067). Details of the phylogenetic tree are shown in Figure 2.

Comparison of genetic diversity of pediatric isolates between China and South Africa

The PCA was used to assess the genetic structure of the pediatric *C. neoformans* AFLP1/VNI isolates from China and South Africa. The genetic structure captured by the first two principal components showed a combined contribution of 77.8%. The pediatric isolates from China clustered into four groups, whereas the pediatric isolates from South Africa clustered into 10 groups. The PCA results agreed with the results of the phylogenetic analysis, which revealed that the ST5 (based on the ISHAM-MLST) isolates from China were closely related to ST9 (based on 11-loci MLST) isolates from South Africa, and ST93 (based on the ISHAM-MLST) isolates from China were closely related to ST8 (based on 11-loci MLST) isolates from South Africa.

The nucleotide diversity (π) of pediatric isolates from South Africa (π=0.00300, n=82) was significantly higher than that from China (π=0.00044, n=25). The average number of nucleotide differences per sequence (k-value) of pediatric isolates from South Africa (k=8.321) was also higher than that of pediatric isolates from China (k=3.493). Together with the pediatric isolates from China and South Africa, the nucleotide sequences of all five loci had polymorphic sites ranging from 1 to 12. Locus IGS1 had the highest nucleotide diversity (π=0.00597) and the highest average number of nucleotide differences per sequence (k=4.307), followed by *LAC1* (π=0.00288, k=1.355) and *GPD1* (π=0.00202, k=1.050). There was a significantly higher percentage of ST31 isolates from Chinese pediatric cases than that from Chinese adult cases (Fisher's Exact test, calibrated *P*=0.036). However, there was no significant difference in ST5 distribution between *C. neoformans* isolates from Chinese pediatric cases and that from Chines adult cases (calibrated *P*=0.183). The details are shown in Table 2, Table 3 and Figure 3.

In vitro antifungal drug susceptibility

The MIC values of QC strain ATCC 6258 in this study were 1 mg/L for AMB, 16 mg/L for 5-FC, 32 mg/L for FLU, 0.5 mg/L for ITR and POS, 0.25 mg/L for VOR and ISA. The MIC values of QC strain ATCC 22019 in this study were 1 mg/L for AMB, 2 mg/L for FLU, 0.125 mg/L for POS, 0.063 mg/L for VOR, 0.031 mg/L for ISA, 0.25 mg/L for 5-FC and ITR. Our results are consistent with what have been reported for these two strains in CLSI M27-A3 and in previous studies.[31-33] The MIC values of the pediatric isolates of the present study were determined for seven antifungal compounds, namely AMB, 5-FC, FLU, ITR, VOR, POS, and ISA. For the 22 isolates, the *in vitro* antifungal MIC_{90} and susceptibility ranges were 0.5 mg/L (0.25-0.50 mg/L) for AMB, 8 mg/L (2-8 mg/L) for 5-FC, 4 mg/L (0.5-8 mg/L) for FLU, 0.125 mg/L (0.031-0.125 mg/L) for VOR, 0.125 mg/L (0.008-0.125 mg/L) for POS, 0.25 μg/mL (0.031-0.250 mg/L) for ITR, and 0.031 mg/L (0.004-0.063 mg/L) for ISA. None of the isolates in this study showed resistance to FLU and 5-FC, which are the two most commonly used antifungal drugs in treatment of cryptococcosis. Five ST5 isolates from Chinese pediatric patients were observed to have decreased susceptibility to ITR, and seven isolates were found to have decreased susceptibility to 5-FC. One ST5 pediatric isolate (SCZ 20172) was observed to have decreased susceptibility to FLU, 5-FC, and ITR. The ST93 and ST53 pediatric isolates in our study were not observed to be resistant or have decreased susceptibility to the seven antifungal compounds. Most pediatric isolates from deceased patients (3/4, 75%) in this study had decreased susceptibility to antifungal compounds. Specifically, two isolates (SCZ 20154 and SCZ 20078) had decreased susceptibility to 5-FC and one isolate had decreased susceptibility to ITR (SCZ 20321). Details are shown in Table 4 and Additional Table S1.

Discussion

Cryptococcosis is a lethal fungal infection that primarily affects adult patients, especially those with AIDS or suffering from other underlying diseases.[3,34] In contrast, cryptococcosis is rare in children, even those who are HIV-infected.[14,35] However, the number of reported cases of cryptococcosis in children has increased in Africa, Europe, South America, and Asia, including China.[15,16,18,36-40] Except for the recent study in South Africa,[37] data on the genotypic diversity and antifungal susceptibility of pediatric *Cryptococcus* isolates is limited.

In this study, cryptococcosis mainly occurred in older children with a mean age of 10.4 ± 3.5 years old, which is similar to that of previous studies.[14,37] One possible explanation for the older onset age is that the amount of cryptococcal exposure is important for cryptococcal infection.[20] Repeated exposure of *C. neoformans* in early childhood may be necessary to establish a latent fungal burden which can be reactivated at older age.[20] Sixty percent of pediatric cryptococcosis patients were male, which is similar to previous studies,[13,14] supporting the theory that *C. neoformans* infections might be gender-related.[40] In terms of infection profiles of pediatric cryptococcosis, the most frequent (84%) symptom was *Cryptococcus* meningitis, followed by disseminated cryptococcosis (12%) and skin lesions (4%). The majority of our *C. neoformans* isolates (80.0%) were recovered from apparently immunocompetent children, and this agrees with previous studies in China (83.6%, 92/110),[13,16,18,40] but differs from studies in USA (20.6%, 13/63)[14] and South Africa (4.4%, 2/45).[36] Earlier investigations suggested that cryptococcosis primarily occurs in apparently immunocompetent adults in China.[21,42] However, in China, AIDS-patients are generally transferred to special hospitals for treatment and the clinical data of these patients are unavailable. Thus, the incidence of cryptococcosis in HIV-positive patients in China might be underestimated.

A considerable percentage of pediatric cryptococcosis cases caused by members of the *C. gattii* species complex has been reported in Colombia (5.9%),[15] South Africa (7.0 %),[43] and Brazil (29.6 %).[44] In contrast, cryptococcal isolates from Chinese pediatric patients were all *C. neoformans* genotype AFLP1/VNI and could be divided into four STs (ST5, ST31, ST53, and ST93), with ST5 as the predominant one. This result is not surprising because ST5 isolate is also predominant among cryptococcal isolates from Chinese adult patients.[1,6] This result might also be related to the predominance of *C. neoformans* AFLP1/VNI in China's natural environment.[45] Notably, our study indicated significant difference in the distribution of ST31 between *C. neoformans* isolates from Chinese pediatric and adult cases (calibrated P=0.036). More studies on the virulence of ST31 isolates are needed to explain the different capability that ST31 isolates showed in infecting Chinese pediatric patients and infecting Chinese adult patients in this study. In addition, all the pediatric isolates were mating type α, which agrees with previous observations that over 90% of both clinical and environmental isolates of *C. neoformans* are mating type α,[8,9] including isolates from China.[42,46] The skewed distribution of *MAT*α isolates might result from clonal expansion by asexual reproduction, self-fruiting, and/or same-sex mating and reproduction among these isolates.[47]

In the present study, low genotypic diversity among isolates obtained from Chinese pediatric patients was observed. Currently, a total of 24 STs of *C. neoformans* have been identified within isolates obtained from Chinese adult patients with predominant sequence type ST5.[6,21,28,46] In our study, only four STs have been identified, and ST5 was also the predominant sequence type (80%, 20/25). This result is reasonable because adult and pediatric patients in China may be exposed to the same natural environment harboring *C. neoformans* isolates. The genotypic diversity among pediatric isolates in China is lower than that observed in South Africa.[37] Both π and k values of the pediatric isolates from South

Africa were higher than those of the isolates from China, which is similar to the results of previous studies.[48,49] Since only five loci sequences were shared between our study and the study from South Africa,[37] phylogenetic analysis and PCA were based on these five shared loci. Phylogenetic analysis suggested that the predominant ST5 (based on the ISHAM-MLST) isolates from China are closely related to ST9 (based on 11-loci MLST) isolates from South Africa, and ST93 (based on the ISHAM-MLST) isolates from China are closely related to the predominant ST8 (based on 11-loci MLST) isolates from South Africa. The PCA results agree with our phylogenetic findings, suggesting significant differences in the population structure of pediatric *Cryptococcus* isolates between China and South Africa. Moreover, we suggest that the ISHAM-MLST scheme should be utilized in investigating the genotypic diversity of more pediatric *C. neoformans* isolates in the future.

The number of reported *C. neoformans* isolates with FLU resistance has increased in the past decade[12] and varies with genotype and geographic region.[31,32] According to the current clinical guidelines for cryptococcosis,[11] AMB and 5-FC were recommended to treat central nervous system (CNS) cryptococcal infection and disseminated cryptococcosis in children and FLU is used in maintenance therapy of cryptococcosis in children. When we apply the recently published ECVs to evaluate the current results, none of the isolates in this study showed resistance to FLU and 5-FC, however, eight ST5 pediatric isolates and two ST31 pediatric isolates showed decreased susceptibility to 5-FC, and/or FLU, and/or ITR. These results agree with the findings of a previous study that showed increasing rates of *C. neoformans* isolates that are resistant to FLU in China, particularly the ST5 isolates.[6] Moreover, most pediatric isolates from deceased patients (3/4, 75%) in this study had decreased susceptibility to antifungal compounds. Two pediatric isolates had decreased susceptibility to 5-FC and one pediatric isolate had decreased susceptibility to ITR,

suggesting that decreased susceptibility to antifungal drugs of *C. neoformans* isolates might be related to therapeutic failure. Thus, antifungal drug susceptibility testing of *C. neoformans* isolates should be recommended to improve prognosis of pediatric cryptococcosis patients.

In summary, the genotypic diversity of pediatric *C. neoformans* isolates from China is low, and the two most frequent sequence types were ST5 and ST31. Decreased susceptibility to FLU, 5-FC, and ITR were observed among pediatric *C. neoformans* isolates from China, particularly among the ST5 isolates. Further investigations of pediatric *C. neoformans* isolates from around the world, particularly relating to genotypic diversity and drug resistance, are needed for better prevention and treatment of pediatric cryptococcosis.

Competing interests

None declared.

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Figure legends

Figure 1.Geographical distribution of pediatric *C. neoformans* isolates from China that were included in this study.

Figure 2. A phylogenetic tree for the representative pediatric *C. neoformans* isolates from China and South Africa using ML and MP methods based on the sequences of five loci (*GPD1*, *LAC1*, IGS1, *PLB1*, and *SOD1*). Sequence types in blue derive from the ISHAM-MLST scheme; sequence types in black derive from 11-loci MLST scheme. ST represents sequence type.

Figure 3. Genetic relationships among *C. neoformans* AFLP1/VNI pediatric isolates from China and South Africa by principal component analysis. Circles represent the isolates from South Africa, and triangles represent the isolates from China. Sequence types in blue derive from the ISHAM-MLST scheme; sequence types in black derive from 11-loci MLST scheme. SA represents South Africa.

Table legends

Table 1. Demographic characteristics of pediatric *C. neoformans* isolates from China

Table 2. Nucleotide polymorphisms in each locus and concatenated sequences of pediatric *C. neoformans* isolates from China and South Africa

Table 3. Comparison of two major ST distributions between pediatric isolates and adult isolates in China

Table 4. The MIC range, MIC_{50}, MIC_{90}, and geometric mean MIC of seven antifungal agents for pediatric *C. neoformans* isolates from China

Additional files

Table S1. Details of the MIC for Chinese pediatric *C. neoformans* isolates for seven antifungals

Authors' contributions

NH, MC, and NX participated in laboratory testing, statistical analysis, and drafting of the manuscript. TB, WHP, FH, JPX, and AMS participated in designing this study and revising the manuscript. WQL and XBZ conceived and designed the study. All of the authors have read and approved the final manuscript.

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Table 1. Demographic characteristics of pediatric *C. neoformans* isolates from China

Isolate	Isolated time	Age	Gender	City	Specimen	Underlying diseases	Outcome	Mating-type	ST	Ref
SCZ 20079	1998-02	11	Male	Fujian	CSF	None	Alive	α	5	This Study
SCZ 20159	1999-02	8	Male	Shanghai	CSF	None	Alive	α	53	This Study
SCZ 20078	1999-11	4	Male	Henan	CSF	None	Died	α	5	This Study
SCZ 20067	2000-12	12	Female	Shanghai	CSF	None	Alive	α	93	This Study
SCZ 20151	2001-02	14	Male	Shanxi	CSF	None	Alive	α	5	This Study
SCZ 20074	2001-04	14	Male	Sichuan	CSF	None	Alive	α	5	This Study
SCZ 20149	2001-06	8	Female	Anhui	CSF	None	Alive	α	5	This Study
SCZ 20072	2001-06	13	Male	Guangdong	CSF	None	Alive	α	5	This Study
SCZ 20098	2001-11	8	Male	Hunan	Skin	None	Alive	α	5	This Study
SCZ 20145	2003-02	8	Female	Hubei	CSF	None	Alive	α	5	This Study
SCZ 20154	2003-08	3	Male	Henan	CSF	None	Died	α	31	This Study
SCZ 20099	2002-05	8	Male	Sichuan	Skin	None	Alive	α	5	This Study
SCZ 20359	2007-04	12	Female	Jiangsu	CSF	None	Alive	α	5	This Study
SCZ 20126	2007-05	6	Female	Shanghai	CSF	None	Alive	α	5	This Study
SCZ 20361	2009-12	14	Male	Beijing	CSF	None	Alive	α	5	This Study
SCZ 20362	2010-05	13	Female	Hubei	CSF	SLE	Alive	α	5	This Study
SCZ 20363	2010-06	7	Male	Hubei	CSF	None	Alive	α	31	This Study
SCZ 20364	2010-08	8	Female	Beijing	Blood	HIV(+)	Alive	α	5	This Study
SCZ 20321	2010-07	15	Female	Sichuan	Blood	HIV(+)	Died	α	5	This Study
SCZ 20322	2010-11	12	Male	Sichuan	CSF	None	Died	α	31	This Study
SCZ 20161	2015-11	15	Male	Zhejiang	CSF	None	Alive	α	5	This Study
SCZ 20172	2016-07	12	Male	Zhejiang	CSF	None	Alive	α	5	This Study
PU 158	2010-08	8	Female	Hebei	Blood	HIV(+)	Alive	ND	5	21
PU 18	2009-12	14	Male	Hebei	CSF	None	Alive	ND	5	21
PU 31	2010-05	13	Female	Shanxi	CSF	SLE	Alive	ND	5	21

CSF: Cerebrospinal fluids

ST: Sequence type

SLE: Systemic Lupus Erythematosus

Table 2. Nucleotide polymorphisms in each locus and concatenated sequences of pediatric *C. neoformans* isolates from China and South Africa

Locus	Pediatric isolates from China			Pediatric isolates from South Africa			Pediatric isolates from China and South Africa			D_{xy}
	S	***π***	***k***	**S**	***π***	***k***	**S**	***π***	***k***	
PLB1	1	0.00015	0.080	6	0.00208	1.106	6	0.00192	1.021	0.00202
LAC1	1	0.00017	0.333	7	0.00298	1.398	7	0.00288	1.355	0.00315
GPD1	1	0.00054	0.280	6	0.00201	1.044	6	0.00202	1.050	0.00231
IGS1	10	0.00388	2.800	12	0.00654	4.714	12	0.00597	4.307	0.00560
SOD1	0	0	0	1	0.00011	0.059	1	0.00008	0.043	0.00006
Concatenated	13	0.00044	3.493	32	0.00300	8.321	32	0.00280	7.776	0.00282

S: number of polymorphic sites.

π: nucleotide diversity.

k: average number of nucleotide differences per sequence.

D_{xy}: average number of nucleotide substitutions per site between populations (pediatric isolates from China vs. pediatric isolates from South Africa).

Table 3. Comparison of two major ST distributions between pediatric isolates and adult isolates in China

Sequence Type	Isolates from pediatric cases (n=25)	Isolates from adult cases (n=162)	*P*-value	Calibrated *P*-value
ST 5	20 (0.800)	145 (0.895)	0.183	0.183
ST 31	3 (0.120)	2 (0.012)	0.018	0.036

Table 4. The MIC range, MIC_{50}, MIC_{90}, and geometric mean MIC of seven antifungal agents for pediatric *C. neoformans* isolates from China

Isolates	Antifungal agents	MIC			
		Range	MIC_{50}	Geometric mean MIC	MIC_{90}
This study (n=22)	AMB	0.25-0.5	0.5	0.441	0.5
	5-FC	2-8	4	4.832	8
	FLU	0.25-8	2	2.130	4
	ITR	0.031-0.25	0.125	0.097	0.25
	VOR	0.031-0.125	0.063	0.069	0.125
	POS	<0.008-0.125	<0.008	0.019	0.125
	ISA	<0.004-0.063	0.015	0.012	0.031